

## Chemo-enzymatic epoxidation of fatty compounds – Focus on processes involving a lipase-catalyzed perhydrolysis step

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The worldwide potential demand for replacing petroleum-derived raw materials with renewable ones for the production of polymeric materials is quite significant from the social and environmental points of view [1]. Beside polysaccharides and sugars, plant oils are the most important renewable raw materials of the chemical industry because of their ready availability [2].

Among the major applications of epoxidized vegetable oils is their use as plasticizer for polyvinyl chloride (PVC) and other plastic materials [3]. Plasticizers are substances that improve flexibility, workability or distensibility of plastics, hence rendering them suitable for diverse applications. Epoxy fatty acids can also be used as PVC-stabilisers because of their ability to slow down degradation by scavenging the free HCl released during PVC decomposition when exposed to heat and light [4]. In addition, epoxidized derivatives of fatty acids can be used as reactive diluents for paints, as intermediates for polyurethane-polyol production, as corrosion protecting agents and as additives to lubricating oils. They also represent valuable raw materials for the production of glues and other surface coatings [5].

Epoxidized oils are currently produced by epoxidation of unsaturated plant oils, such as soybean or linseed oil. Although several methods are available to epoxidize the double bonds of unsaturated fatty acids, the only method applied on industrial scale is the Prile-

**Abstract:** At the industrial scale, the chemical Prileshajev reaction is currently the only method applied to produce epoxidized plant oils. Using enzymes could be an alternative way allowing an environmentally benign and more selective epoxidation reaction. P450 monooxygenases, diiron-center oxygenases, lipoxygenases, peroxygenases, and hydrolases performing perhydrolysis are enzyme classes involved in free fatty acid and glyceride epoxidation. After a brief description of these biocatalysts, this review focuses on the chemo-enzymatic epoxidation of unsaturated fatty acid chains, where a lipase-catalyzed peroxy acid formation is followed by an uncatalyzed "self-epoxidation". The molecular bases of lipase-catalyzed perhydrolysis as well as the different parameters influencing the epoxidation reaction are reviewed and described in details.

**Key words:** epoxidized plant oils, cytochromes P450, diiron-center oxygenases, lipoxygenases, peroxygenases, lipases, perhydrolysis

shajev epoxidation reaction. In this reaction, short chain peroxy acids such as peracetic acid are generated from the corresponding acid and hydrogen peroxide in the presence of a strong mineral acid. Then, these peroxy acids react with unsaturated fatty acid C=C double bonds to obtain epoxidized fatty acids. Peroxy acids are prepared either in a separate step or *in situ*. Due to the potential danger of handling peroxy acids, the *in situ* method is generally preferred for large-scale epoxidation of unsaturated triglycerides [6]. However, this chemical method for epoxidation has several disadvantages. First of all, there are considerable side reactions *via* oxirane ring opening, leading to diols, hydroxyesters, estolides and other dimers, which are believed to be catalyzed by the presence of a strong mineral acid [7]. As a result, the selectivity of this process never exceeds 80% [6]. Furthermore, the presence of a strong acid in an oxidative environment causes equipment corrosion problems. Finally, this acid must be recycled or neutralized before discharge into the environment. Using enzymes is an alternative solution that allows an environmentally benign and more selective epoxidation reaction.

### Main enzymes involved in epoxidation

Several enzyme types are directly or indirectly involved in fatty acid epoxidation: cytochrome

P450 monooxygenases, diiron-center oxygenases, lipoxygenases, peroxygenases, perhydrolyases and lipases. They belong to the oxydoreductase (EC 1.x.x.x) or hydrolase (EC 3.x.x.x) enzyme classes.

### Oxydoreductases

Cytochrome P450 monooxygenases (EC 1.14.x.y) are one of the largest and oldest enzyme superfamilies with several thousand sequences reported up to now [5, 8]. Cytochromes P450 contain a heme-thiolate prosthetic group. They are widely distributed in animals, plants, fungi and bacteria and catalyze a vast range of monooxygenation reactions in catabolic and anabolic pathways [9, 10]. P450s incorporate a single atom of molecular oxygen into a substrate with the concomitant reduction of the other atom to water. With respect to fatty acids, they can act both as hydroxylases and as epoxidases of unsaturated fatty acids. The reducing equivalents are delivered by the nicotinamide cofactor NAD(P)H. As this cofactor is far too expensive for industrial applications, one major challenge for all attempts to construct a bioreactor with isolated P450 enzymes is to engineer an artificial electron supply system, generally consisting in a coupling with a deshydrogenase-catalyzed NAD(P) reduction [11]. CYP102A1, also called P450 BM-3, is one of the most intensely studied P450 monooxygenases. This 119 kDa enzyme,

originally cloned from *Bacillus megaterium*, is a self-sufficient P450 in which an FAD- and FMN-containing reductase and the P450-domain are naturally fused on a single peptide chain [12]. The experimental setup for its application in organic synthesis is a lot easier compared to other P450s which require one or two additional electron transport proteins for activity. Indeed, in the presence of NADPH and O<sub>2</sub> P450 BM-3 can catalyze the oxygenation of long chain fatty acids without the aid of any other protein. Moreover, all self-sufficient P450 monooxygenases characterized to date exhibit rather high turnover numbers (> 1,000 s<sup>-1</sup>) with their preferred substrates [5].

Diiron-center oxygenases are mainly present in plants and bacteria. They are often referred to as "diiron-oxo" proteins because most proteins containing a binuclear diiron cluster react with dioxygen as part of their functional processes [13]. These proteins catalyze diverse reactions including hydroxylation, epoxidation, and desaturation according to the following catalytic mechanism: reductive oxygen is activated by the diiron-center, a hydrogen atom from a CH-bond is then abstracted and the oxygen atom is incorporated into the substrate via a radical rebound mechanism [5, 14]. Diiron-center oxygenases and desaturases share high sequence homology. From a biotechnological point of view, diiron-oxo oxygenases present the same disadvantages that P450 enzymes as both enzyme classes use electrons originating from the costly cofactor NAD(P)H for reductive oxygen activation and require an electron transport chain delivering these reduction equivalents. That is why, despite their occurrence in most plant species producing oils enriched in epoxy acids, attempts to exploit diiron cluster-containing fatty acid oxygenases in biotechnology are still scarce.

Lipoxygenases (EC 1.13.11.12; LOXs) constitute a large gene family of non-heme iron containing fatty acid dioxygenases, which are ubiquitous in plants and animals. They catalyze the regio- and stereospecific incorporation of dioxygen into polyunsaturated fatty acids

(linoleic acid,  $\alpha$ -linolenic acid or arachidonic acid) to generate optically active (S)-dienic hydroperoxides [15-17]. Plant lipoxygenases are classified according to their regioselectivity using linoleic acid as substrate. Linoleic acid is oxygenated either at the  $\Delta^9$  carbon atom (9-LOX) or at the  $\Delta^{13}$  carbon atom (13-LOX) of the hydrocarbon backbone of the fatty acid leading to the formation of two groups of compounds: the (9S)-hydroperoxy- and the (13S)-hydroperoxy derivatives (figure 1). For example, soybean LOX isoenzyme-1 is classified as a 13-LOX whereas potato tuber LOX is classified as a 9-LOX [5]. Both the cavity within the active site and the orientation of the substrate are important determinants for the regiospecificity of plant lipoxygenases [18]. The lipid hydroperoxides formed by lipoxygenases are highly reactive and are immediately used by plant cells for the biosynthesis of a wide range of compounds including epoxides. The metabolism of oxidized polyunsaturated fatty acids via the lipoxygenase-catalyzed step and the subsequent reactions are collectively called the LOX pathway [16, 19, 20]. Concerning biotechnological applications, the use of isolated lipoxygenases in organic solvents and their immobilization have up to now been poorly investigated [21].

Peroxygenases (EC 1.14.x.y) are P450-related enzymes of the LOX pathway that use the hydroperoxides formed by lipoxygenases as substrate. These enzymes are membrane-bound proteins containing heme b as prosthetic group [22] and are ubiquitous in plants. The first reactions described for peroxygenases are co-oxidative reactions such as epoxidation, hydroxylation, and sulfoxidation, where an oxygen atom from the hydroperoxide is directly transferred to the substrate. When using hydroperoxides deriving from unsaturated fatty acids, this oxygen transfer can also occur via an intramolecular mechanism [23]. Peroxygenases do not require cofactors such as NAD(P)H or FAD. In the presence of an organic hydroperoxide, oleic acid is converted into the corresponding 9,10-epoxide by peroxygenase

activities isolated from soybean, broad bean and oat [23-25]. Piazza *et al.* [26, 27] have developed a method for the rapid isolation and immobilization of peroxygenases on membranes, and conducted epoxidation reactions in organic solvents.

## Hydrolases

Hydrolases such as lipases (EC 3.1.1.3) and carboxyl esterases (EC 3.1.1.1) have been shown to produce peroxy acids from hydrogen peroxide and fatty acids by perhydrolysis reaction. These peroxy acids subsequently epoxidize unsaturated fatty acids via an uncatalyzed reaction. In this case, large-scale applications seem to be possible in the near future. The next chapter of this review focuses on lipase-catalyzed perhydrolysis.

## Lipase-catalyzed perhydrolysis

### Chemo-enzymatic epoxidation of oils and fats

In 1990, an immobilized form of lipase B from *Candida antarctica* (Novozyme 435) was shown to catalyze the conversion of saturated fatty acids into peroxy fatty acids in the presence of hydrogen peroxide [28]. Indeed, perhydrolysis, where hydrogen peroxide acts as the nucleophile instead of water in the deacylation step, might be a side activity of many serine hydrolases. However, the perhydrolyase activity of lipases and esterases is generally much lower than their esterase activity and some of them, such as subtilisin, do not exhibit perhydrolyase activity [29]. To the contrary, some hydrolases display a higher perhydrolysis activity than hydrolysis and are therefore described as perhydrolyases [30, 31]. Protein engineering has also been used to turn hydrolases into perhydrolyases [32-34].

The lipase-mediated synthesis of peroxy acids from carboxylic acids and hydrogen peroxide can be used to perform *in situ* epoxidation of alkenes [28, 35]. When unsaturated fatty acids or their esters are treated with hydrogen peroxide in the presence of an enzyme such as Novozyme 435, epoxidized derivatives are produced [36]. The reaction proceeds in two steps (figure 2). Firstly, unsaturated fatty acids are converted into unsaturated peroxy acids by lipase-catalyzed perhydrolysis. Unsaturated peroxy or carboxylic acids are then epoxidized via an uncatalyzed Prileshajev reaction that is often referred to as "self-epoxidation reaction" in spite of the fact that it proceeds predominantly via an intermolecular process [36]. More recently, a lipase from *Pseudomonas* sp. was shown to catalyze the epoxidation of cholesterol and stigmaterol in the presence of

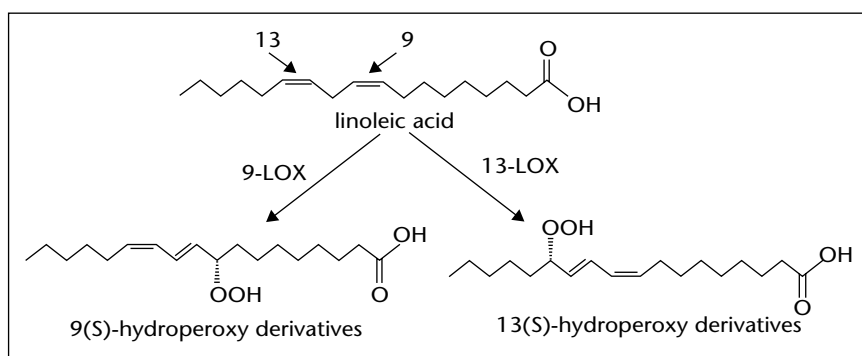


Figure 1. Lipoxygenase reaction and regiospecificity.

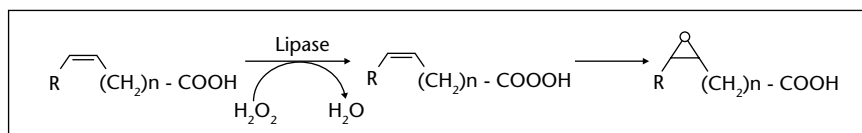


Figure 2. Chemo-enzymatic epoxidation of unsaturated fatty acids involving a lipase-catalyzed perhydrolysis step.

high hydrogen peroxide concentration (50%) and ethyl acetate, while olefins were converted into diols in the reaction conditions tested [37]. In addition to the production of partially or completely epoxidized free fatty acids, the chemo-enzymatic method can also be applied to produce epoxidized plant oils [6, 38, 39]. If a triglycerol embodying unsaturated fatty acids is treated with  $H_2O_2$  in the presence of a suitable lipase, peroxy fatty acids are formed that epoxidize the C=C double bonds. The resulting mixture contains epoxidized triglycerides, a small amount of epoxidized free fatty acids, epoxidized mono- and diglycerides. The separation of these mono- and diglycerides from the reaction medium is difficult. Although there may be applications where these by-products do not matter, a way to prevent their formation has been found by adding free fatty acids to the starting material [38]. In this way, perhydrolysis occurs but all the hydroxyl groups of glycerol are reesterified by the excess of free fatty acids. The resulting product only consists of epoxidized triglycerides and epoxidized free fatty acids that can be easily removed by alkaline washing if necessary. Using this method, rapeseed, sunflower, soybean and linseed oil were epoxidized with conversions and selectivities well above 90% [6].

Chemo-enzymatic epoxidation is of considerable interest because this method occurs in mild conditions and suppresses undesirable ring opening of the epoxide. The epoxidation processes described by Rüscher *et al.* [6] have already been carried out on the kilogram scale. If further successful, the lipase-catalyzed perhydrolysis could replace the problematic chemical Prileshajev epoxidation on industrial scale.

#### Molecular approach of lipase-catalyzed perhydrolysis

The crystal structure of *Candida antarctica* lipase B shows that this enzyme has a Ser-His-Asp catalytic triad in its active site. The structure appears to be in an "open" conformation with a rather restricted entrance to the active site that explains the substrate specificity and the high degree of stereospecificity of this lipase. In addition, the low hydrolytic activity towards triglycerides of long chain fatty acids is also probably due to the narrow and deep active site of the enzyme [40]. Cofactor-free haloperoxidases, also called perhydrolases,

contain a Ser-His-Asp catalytic triad analogous to that reported for lipases [41]. Both groups of enzymes seem to share a common origin and an analogous catalytic mechanism, in which the involvement of the catalytic triad would be crucial [41, 42]. Perhydrolysis presumably occurs with an esterase-like mechanism: a carboxylic acid first reacts with the active site serine group to form an acyl-enzyme intermediate, which reacts with hydrogen peroxide to form a peroxy acid. Nevertheless the Ser-His-Asp catalytic triad is not the only determinant for perhydrolase activity because some serine hydrolases don't have this activity [29]. An alternate mechanism has been proposed for perhydrolysis in which the catalytic serine stabilizes the carboxylic acid substrate with a hydrogen bond instead of forming an acyl-enzyme intermediate [43]. However there is currently no experimental evidence for this proposal. An explanation proposed to elucidate the difference in activities between hydrolases and perhydrolases concerns the electro-negative microenvironment of the active site. The more hydrophobic environment present in perhydrolases compared to other hydrolases would protect the peroxy acid against hydrolysis [44]. Besides, the presence in the structure of the enzyme of amino acids particularly sensitive to oxidation by  $H_2O_2$  and by peroxy acids can also explain the difference in enzymatic activities [41].

Recently, Bernhardt *et al.* [29] performed the alignment of the amino acid sequences of six hydrolases and six perhydrolases in order to observe which residues appeared in perhydrolases but not in esterases. The differing amino acids within a sphere of 12 Å around the reactive hydroxyl of the catalytic serine were mutated by molecular biology techniques. The substitution of a single amino acid was sufficient to shift the hydrolase activity of an aryl esterase from *Pseudomonas fluorescens* to make perhydrolysis the preferred reaction in aqueous solution. A molecular basis for the increase in perhydrolase activity is the presence of a hydrogen bond formed between a carbonyl oxygen atom of the enzyme and the peroxide nucleophile. This peroxide hydroxy-carbonyl hydrogen bond stabilizes the hydrogen peroxide attack on the putative acyl-enzyme intermediate, hence facilitating the perhydrolysis reaction [29].

#### Reaction parameters influencing the chemo-enzymatic epoxidation

The applicability of chemo-enzymatic epoxidation using the lipase B from *Candida antarctica* has been demonstrated with different substrates such as several fatty acids, fatty acid esters including vegetable oils, and other olefins [36, 38, 45].

Chemo-enzymatic epoxidation is influenced by various reaction parameters. Hydrogen peroxide concentration is the most critical parameter influencing the reaction rate and the degree of epoxidation. Orellana-Coca *et al.* [46] showed that an excess of hydrogen peroxide compared to the amount of double bonds is necessary in order to yield a total conversion of linoleic acid within a short time period. However, a large excess of hydrogen peroxide results in the accumulation of peroxy acids in the final product. These unreacted peroxy acids could be a potential problem for reasons of safety and contamination of the final product. Moreover, a high hydrogen peroxide concentration in the reaction medium negatively affects enzyme activity [46]. The gradual addition of hydrogen peroxide could be a solution to reduce the deactivation of the biocatalyst [28, 35, 46].

Increasing reaction temperature has a positive effect on the reaction rate of chemo-enzymatic epoxidation but it should be below 50°C to avoid hydrogen peroxide decomposition and possible enzyme inactivation [46].

Most of the investigations have involved dilution of the substrate in an organic solvent, particularly in toluene. Recently, lipase-mediated epoxidation in a more environmentally-friendly, solvent-free medium has also been reported. In these conditions, reaction temperature has a significant impact on epoxidation. A study concerning the chemo-enzymatic epoxidation of linoleic acid [46] showed that the reaction in a solvent-free medium is not complete at 30°C due to the formation of a solid or a highly viscous oily phase, creating mass transfer limitations. Increasing the temperature up to 60°C and using some excess of hydrogen peroxide helped in improving the rate of epoxide formation. In another study, the chemo-enzymatic epoxidation of oleic acid and its methyl ester under solvent-free conditions was characterized [47]. Epoxystearic acid and epoxystearic acid methyl ester were synthesized with very good yields.

#### Stability of lipase during chemo-enzymatic epoxidation

Enzyme cost is among the important factors determining the economics of a biocatalytic process. To make chemo-enzymatic processes competitive with chemical processes, a high



enzyme stability and the possibility to recycle the enzyme are highly desirable. In an investigation by Warwel *et al.* involving epoxidation in a toluene containing reaction medium it was found that *Candida antarctica* B lipase (Novozyme 435) was very stable, with 75% of residual activity after 15 reaction cycles [36]. Nevertheless, when chemo-enzymatic reaction occurred in a solvent-free medium under conditions optimized for achieving high reaction rates and epoxidized product yields, the enzyme was found to suffer loss in activity, hence limiting its recycling [47].

Two parameters are harmful for the activity and for operational stability of lipase B from *C. antarctica* (Novozyme 435) in chemo-enzymatic epoxidation: hydrogen peroxide at high concentrations together with elevated temperatures. Indeed, in the presence of 6-12M hydrogen peroxide, the enzyme is rather stable at 20°C whereas at 60°C the enzyme loses activity rapidly. The rate of deactivation increases with increasing H<sub>2</sub>O<sub>2</sub> concentration [48]. For epoxidation processes run at elevated temperatures, a controlled addition of H<sub>2</sub>O<sub>2</sub> is hence important for enzyme stability. In an industrial chemo-enzymatic process, temperature control and careful dosage of hydrogen peroxide would be essential to optimize the enzyme stability.

## Conclusion and outlook

Despite the fact that fatty acid-epoxidizing enzymes are versatile and useful biocatalysts, their industrial applications are still scarce. Cytochrome P450 monooxygenases and diiron cluster-containing monooxygenases offer an interesting possibility for fatty acid oxidation but up to now this is an issue of academic research only. An efficient method for the substitution of their expensive cofactors NAD(P)H has to be found in order to use these biocatalysts in preparative synthesis. Currently, biocatalytic epoxidation of fatty acids using isolated enzymes is limited to the use of lipoxygenases, peroxygenases and lipase-catalyzed perhydrolysis followed by "self-epoxidation". The information provided in this review focused on the lipase-catalyzed perhydrolysis. The recent understanding of some molecular bases of this catalytic performance as well as the development of directed evolution techniques, eventually in combination with rational enzyme engineering, open up the possibility to overcome problems yet unresolved. For example, this approach could lead to enzymes more resistant to the drastic reaction conditions originating from the use of hydrogen peroxide in high concentrations. Enhancing the perhydrolysis/hydrolysis rate ratio and improving enzyme stability to favour its recycling

could reduce the cost of the process. In this way the chemo-enzymatic epoxidation of unsaturated fatty acids seems to be possible on industrial scale to replace the Prileshajev chemical reaction.

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